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## A Third-Generation Lentivirus Vector with a Conditional Packaging System

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Vectors derived from human immunodeficiency virus (HIV) are highly efficient vehicles for *in vivo* gene delivery. However, their biosafety is of major concern. Here we exploit the complexity of the HIV genome to provide lentivirus vectors with novel biosafety features. In addition to the structural genes, HIV contains two regulatory genes, *tat* and *rev*, that are essential for HIV replication, and four accessory genes that encode critical virulence factors. We previously reported that the HIV type 1 accessory open reading frames are dispensable for efficient gene transduction by a lentivirus vector. We now demonstrate that the requirement for the *tat* gene can be offset by placing constitutive promoters upstream of the vector transcript. Vectors generated from constructs containing such a chimeric long terminal repeat (LTR) transduced neurons *in vivo* at very high efficiency, whether or not they were produced in the presence of Tat. When the *rev* gene was also deleted from the packaging construct, expression of *gag* and *pol* was strictly dependent on Rev complementation *in trans*. By the combined use of a separate nonoverlapping Rev expression plasmid and a 5' LTR chimeric transfer construct, we achieved optimal yields of vector of high transducing efficiency (up to 10<sup>7</sup> transducing units [TU]/ml and 10<sup>4</sup> TU/ng of p24). This third-generation lentivirus vector uses only a fractional set of HIV genes: *gag*, *pol*, and *rev*. Moreover, the HIV-derived constructs, and any recombinant between them, are contingent on upstream elements and *trans* complementation for expression and thus are nonfunctional outside of the vector producer cells. This split-genome, conditional packaging system is based on existing viral sequences and acts as a built-in device against the generation of productive recombinants. While the actual biosafety of the vector will ultimately be proven *in vivo*, the improved design presented here should facilitate testing of lentivirus vectors.

Lentiviruses have attracted the attention of gene therapy investigators (45) for their ability to integrate into nondividing cells (8, 15, 16, 25, 26). We previously developed replication-defective vectors from the lentivirus human immunodeficiency virus (HIV) and showed that they transduce target cells independent of mitosis (32). The vectors proved highly efficient for *in vivo* gene delivery and achieved stable long-term expression of the transgene in several target tissues, such as the brain (5, 33), the retina (31), and the liver and muscle of adult rats (21). A major concern, however, is the biosafety of vectors derived from a highly pathogenic human virus.

The complexity of the lentivirus genome may be exploited to build novel biosafety features in the design of a retrovirus vector. In addition to the structural *gag*, *pol*, and *env* genes common to all retroviruses, HIV contains two regulatory genes, *tat* and *rev*, essential for viral replication, and four accessory genes, *vif*, *vpr*, *vpu*, and *nef*, that are not crucial for viral growth *in vitro* but are critical for *in vivo* replication and pathogenesis (27).

The Tat and Rev proteins regulate the levels of HIV gene expression at transcriptional and posttranscriptional levels, respectively. Due to the weak basal transcriptional activity of the HIV long terminal repeat (LTR), expression of the provirus initially results in small amounts of multiply spliced transcripts coding for the Tat, Rev, and Nef proteins. Tat increases dramatically HIV transcription by binding to a stem-loop structure (transactivation response element [TAR]) in the nascent RNA,

thereby recruiting a cyclin-kinase complex that stimulates transcriptional elongation by the polymerase II complex (46). Once Rev reaches a threshold concentration, it promotes the cytoplasmic accumulation of unspliced and singly spliced viral transcripts, leading to the production of the late viral proteins. Rev accomplishes this effect by serving as a connector between an RNA motif (the Rev-responsive element [RRE]), found in the envelope coding region of the HIV transcript, and components of the cell nuclear export machinery. Only in the presence of Tat and Rev are the HIV structural genes expressed and new viral particles produced (27).

In a first generation of HIV-derived vectors (32), viral particles were generated by expressing the HIV type 1 (HIV-1) core proteins, enzymes, and accessory factors from heterologous transcriptional signals and the envelope of another virus, most often the G protein of the vesicular stomatitis virus (VSV G) (9) from a separate plasmid. In a second version of the system, the HIV-derived packaging component was reduced to the *gag*, *pol*, *tat*, and *rev* genes of HIV-1 (51). In either case, the vector itself carried the HIV-derived *cis*-acting sequences necessary for transcription, encapsidation, reverse transcription, and integration (2, 4, 22, 24, 29, 30, 32, 35). It thus encompassed, from the 5' to 3' end, the HIV 5' LTR, the leader sequence and the 5' splice donor site, approximately 360 bp of the *gag* gene (with the *gag* reading frame closed by a synthetic stop codon), 700 bp of the *env* gene containing the RRE and a splice acceptor site, an internal promoter (typically the immediate-early enhancer/promoter of human cytomegalovirus [CMV] or that of the phosphoglycerokinase gene [PGK]), the transgene, and the HIV 3' LTR. Vector particles are produced by cotransfection of the three constructs in 293T cells (32). In this design, significant levels of transcription from the vector

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LTR and of accumulation of unspliced genomic RNA occur only in the presence of Tat and Rev.

Here, we demonstrate that the *trans*-acting function of Tat becomes dispensable if part of the upstream LTR in the transfer vector construct is replaced by constitutively active promoter sequences. Furthermore, we show that the expression of rev in *trans* allows the production of high-titer HIV-derived vector stocks from a packaging construct which contains only gag and pol. This design makes the expression of the packaging functions conditional on complementation available only in producer cells. The resulting gene delivery system, which conserves only three of the nine genes of HIV-1 and relies on four separate transcriptional units for the production of transducing particles, offers significant advantages for its predicted biosafety.

#### MATERIALS AND METHODS

Transfer vector constructs, pHR'CMV-LacZ and pHR'CMV-Luciferase have been described elsewhere (32). pHR2 is a lentivirus transfer vector in which the polylinker and downstream nef sequences up to the KpnI site of pHR' have been replaced with a *Cla*I-*Spe*I-*Sma*I-*Bam*H-*Sac*I-*Eco*R I polylinker. pHR2 was generated by replacing the 3.7-kb *Cla*I-*Sac*I fragment of pHR'CMVlacZ with a 697-bp *Cla*I-*Sac*I fragment generated by PCR using pHR'CMVlacZ as the template with oligonucleotide primers 5'-CCAUCUAGGACTAGTCTACGIVATCCTGGGGACGGTATCGCGGAATTCTGTTAAAGACCAATGAC-3' and 5'-TTATAATGTCAGGCCCTCTG-3', followed by digestion with *Cla*I and *Sac*I.

pHR2PGK-NGFR, pHR2CMV-NGFR, and pHR2MFG-NGFR are lentivirus transfer vectors in which the truncated low-affinity nerve growth factor receptor (NGFR) (6) transgenes under the control of the murine PGK, human CMV, and Moloney leukemia virus (MLV) promoters, respectively, have been inserted into the polylinker of pHR2. The pHR2PGK-NGFR transgene encodes no intron sequences. The pHR2CMV-NGFR vector includes the intron from plasmid pMD (34), and the pHR2MFG-NGFR vector contains the MLV intron from MFG-S (34).

pRLL, pRLL, pCCL, and pCLL are lentivirus transfer vectors containing chimeric Rous sarcoma virus (RSV)-HIV or CMV-HIV 5' LTRs and vector backbones in which the simian virus 40 polyadenylation and (enhancers) origin of replication sequences have been included downstream of the HIV 3' LTR, replacing most of the human sequences remaining from the HIV integration site. In pRLL, the enhancer and promoter (nucleotides -233 to -1 relative to the transcriptional start site; GenBank accession no. JU2342) from the U3 region of RSV are joined to the R region of the HIV-1 LTR. In pRLL, the RSV enhancer (nucleotides -233 to -50) sequences are joined to the promoter region (from position -78 relative to the transcriptional start site) of HIV-1. In pCCL, the enhancer and promoter (nucleotides -673 to -1 relative to the transcriptional start site; GenBank accession no. KU2114) of CMV were joined to the R region of HIV-1. In pCLL, the CMV enhancer (nucleotides -673 to -230) was joined to the promoter region (position -78) of HIV-1. Exact sequences and details of construction are available on request.

pHR2hPGK-GFP, pCCLhPGK-GFP, pCLLhPGK-GFP, pRLLhPGK-GFP, and pRLLhPGK-GFP are lentivirus transfer vectors containing the enhanced green fluorescent protein (eGFP) (750-bp *Bam*H-*Nsi*I fragment from pEGFP-1; Clontech) coding region, under the control of the human PGK promoter (nucleotides 5 to 516; GenBank accession no. M11958), inserted into the polylinker region of each parental vector. pRLLhPGK-GFP was obtained by deletion of the *Xba*I-*Bam*H fragment containing the PGK promoter from pRLLhPGK-GFP.

pRLLhPGK-GFP.SIN-18 is a vector in which 5' LTR sequences from -418 to -18 relative to the U3/R border have been deleted from pRLLhPGK-GFP (57).

Packaging constructs. The *lacZ*-defective packaging construct pCMVΔR.95 was obtained by swapping an *Eco*R-*Sac*I fragment from plasmid R7/pac(+) (12) with the corresponding fragment of pCMVΔR.91, a previously described plasmid expressing Clag, Pol, Tat, and Rev (51). This fragment has deletion affecting the initiation codon of the *lacZ* gene and a frameshift created by the insertion of an *Mst*I linker into the *Bsu*3I site as described previously. pCMVΔR.74 is a derivative of pCMVΔR.91 in which a 153-bp *Sac*II fragment, containing a splice donor site, has been deleted from the CMV-derived region upstream of the HIV sequences to optimize expression.

pMDLg/p is a CMV-driven expression plasmid that contains only the gag and pol coding sequences from HIV-1. First, pMDLg/p was constructed by ligating a 4.2-kb *Cla*-*Eco*R fragment from pCMVΔR.74 with a 3.3-kb *Eco*R-*Hind*III fragment from pkar2 (14) and a 0.9-kb *Hind*III-*Nco*I fragment from pkar2 along with an *Nco*-*Cla*I linker consisting of synthetic oligonucleotides 5'-CATGGGT GCGAAGGCGTCAGTATAAGCGGGGGAGAATTAGAT-3' and 5'-CG ATCTAATTCCTCCCCGCTTAACTGAGCCTCTCGCACC-3'. Next, pMDLg/p was constructed by inserting the 4.25-kb *Eco*R fragment from pkar2g/p into the *Eco*R site of pMD-2. pMD-2 is a derivative of pMD.G (34) in which the pXFR3 plasmid backbone of pMD.G has been replaced with a

minimal pUC plasmid backbone and the 1.6-kb VSV G-encoding *Eco*R fragment has been removed.

pMDLg/pRRE differs from pMDLg/p by the addition of a 374-bp RRE-containing sequence from HIV-1 (HXBc) immediately downstream of the *pol* coding sequence. To generate pMDLg/pRRE, the 374-bp *Nsi*-*Hind*III RRE-containing fragment from pHR3 was ligated into the 9.3-kb *Nsi*-*Bgl*II fragment of pVL1393 (Invitrogen) along with a *Hind*III-*Bgl*II oligonucleotide linker consisting of synthetic oligonucleotides 5'-AGCTTCCGCGGA-3' and 5'-GATCTCC GCGGA-3' to generate pVL1393RRE (pHR3 was derived from pHR3 by the removal of HIV *env* coding sequences upstream of the RRE sequences in pHR3). A *Nsi* site remains at the junction between the gag and RRE sequences. pMDLg/pRRE was then constructed by ligating the 380-bp *Eco*R-*Sst*I fragment from pVL1393RRE with the 3.15-kb *Sst*I-*Nde*I fragment from pMD-2F1X (pMD-2F1X is a human factor IX-containing variant of pMD-2 which has an *Sst*I site at the 3' end of the factor IX insert), the 2.25-kb *Nde*-*Av*I fragment from pMDLg/p, and the 3.49-kb *Av*I-*Eco*R fragment from pXFLg/p (14).

pRSV-Rev and pTK-Rev (generous gifts of T. Hope, Salt Institute) are Rev CDNA-expressing plasmids in which the joined second and third exons of HIV-1 rev are under the transcriptional control of the RSV U3 and herpes simplex virus type 1 thymidine kinase (TK) promoters, respectively. Both expression plasmids utilize polyadenylation signal sequences from the HIV LTR in a pUC118 plasmid backbone.

Vector production and assays. Vectors were produced by transfecting into 293T cells as previously described (33), with the following modifications. A total of  $5 \times 10^6$  293T cells were seeded in 10-cm-diameter dishes 24 h prior to transfection in Iscove modified Dulbecco culture medium (JRH Biosciences) with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) in a 5% CO<sub>2</sub> incubator, and the culture medium was changed 2 h prior to transfection. A total of 20  $\mu$ g of plasmid DNA was used for the transfection of one dish: 3.5  $\mu$ g of the envelope plasmid pMD.G, 6.5  $\mu$ g of packaging plasmid, and 10  $\mu$ g of transfer vector plasmid. The precipitate was formed by adding the plasmid to a final volume of 450  $\mu$ l of 0.1  $\times$  TE (10 mM Tris [pH 8.0] plus 1 mM EDTA) and 50  $\mu$ l of 2.5 M CaCl<sub>2</sub>, mixing well, then adding dropwise 500  $\mu$ l of 2X HEPES-buffered saline (281 mM NaCl, 10 mM HEPES, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub> [pH 7.12]) while vortexing and immediately adding the precipitate to the cultures. The medium (10 ml) was replaced after 14 to 16 h; the conditioned medium was collected after another 24 h, cleared by low-speed centrifugation, and filtered through 0.22- $\mu$ m-pore-size cellulose acetate filters. For *in vitro* experiments, serial dilutions of freshly harvested conditioned medium were used to infect 10<sup>5</sup> cells in a six-well plate in the presence of Polybrene (8  $\mu$ g/ml). Viral p24 antigen concentration was determined by immunospot (Alliance; DuPont-NEN). Vector batches were tested for the absence of replication-competent virus by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for 3 weeks. In all cases tested, p24 was undetectable (detection limit, 3 pg/ml) once the input antigen had been eliminated from the culture. Transducing activity was expressed in transducing units (TU).

Northern blot analysis. Total RNA was isolated from  $1 \times 10^7$  to  $2 \times 10^7$  cells harvested at confluence by using RNAzol B as suggested by the manufacturer; 10 to 20  $\mu$ g of RNA was loaded per well on 1% agarose gels, using NorthernMax (Ambion, Austin, Tex.) reagents as described by the manufacturer. Transfer was to ZetaBind membranes (Cuno Inc., Meriden, Conn.) by either capillary transfer or pressure blotting (Stratagene). <sup>32</sup>P-labeled probes were made by random priming.

Intracerebral injection of vectors. Twelve Fischer 344 male rats weighing approximately 220 g were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). The rats were housed with access to ad libitum food and water on a 12-h light/dark cycle and were maintained and treated in accordance with published National Institutes of Health guidelines. All surgical procedures were performed with the rats under isoflurane gas anesthesia, using aseptic procedures. After a rat was anesthetized in a sleep box, it was placed in a small animal stereotaxic device (Kopf Instruments, Tujunga, Calif.) using the earbars, which do not break the tympanic membrane. The rats were randomly divided into one control and four treatment groups. After the rats were placed in the stereotaxic frame, 2  $\mu$ l of lentivirus vector concentrated by ultracentrifugation at 50,000  $\times$  g for 140 min at 20°C (38) in phosphate-buffered saline (PBS) was injected consecutively into the striatum in both hemispheres over 4 min at a rate of 0.5  $\mu$ l/min (coordinates, AP 0.0, LAT  $\pm$ 3.0, DV 5.5, -4.5, 3.5 with the injector bar set at 3.3 mm below the intra-aural line [56]), using a continuous-infusion system as described previously in detail (28). During the injection, the needle was slowly raised 1 mm in the dorsal direction every 40 s (3-mm total withdrawal). One minute after cessation of the injection, the needle was retracted an additional 1 mm and then left in place for an additional 4 min before being slowly withdrawn from the brain.

Histology. One month after vector injection, each animal was deeply anesthetized with intraperitoneal pentobarbital and perfused through the aorta with sterile PBS, followed by ice-cold 4% paraformaldehyde perfusion. The brains were removed from the skulls, postfixed in 4% paraformaldehyde by immersion for 24 h, and then transferred into a 30% sucrose-PBS solution for 3 to 4 days, until the brains sank to the bottom of their containers. The brains were then frozen on dry ice, and 40- $\mu$ m-thick coronal sections were cut on a sliding microtome. Sections were collected in series in microtiter well plates that contained a glycine-based antifreeze solution, and they were kept at -80°C until further processing. Immunocytochemistry was performed according to the general pro-

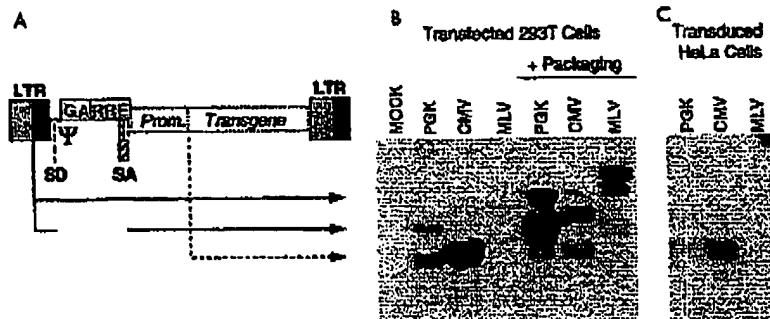


FIG. 1. Northern analysis of the RNA expression from lentivirus vectors. Three pH2R vectors carrying an expression cassette for the same transgene (truncated low-affinity NGF-R) and driven by three different promoters (PGK, CMV, and retroviral MFG) were analyzed in producer and transduced cells. Total RNA was extracted and analyzed by Northern blotting with a probe specific for the transgene sequence. (A) Schematic of the vector construct depicts the species of RNA driven by the internal promoter (Prom.; broken arrow, shorter transcript) and the viral LTR (solid arrow, longer transcript) due to the splicing of the viral intron. The splice donor and acceptor sites (SD and SA), the packaging sequences (Ψ), the truncated gag sequence (GA), and the RRE are indicated. (B) The vector constructs were transfected in 293T cells without or with the packaging construct. (C) Vector particles produced by the 293T transfectants were used to transduce HeLa cells. In the absence of the viral transactivators, supplied by the core packaging construct only in the producer cells, vector expression occurs mainly from the internal promoter. Note the dramatic enhancement of the upstream transcription and the accumulation of unspliced RNA (carrying the Ψ sequence) in the presence of the packaging construct. In the transduced cells, the LTR is silenced. Note that the three expression cassettes differ in the size of the promoters and 5' untranscribed sequence. In each case, the smallest RNA species represents transcripts initiated from the internal promoter, while the intermediate-size and larger species correspond to spliced and unspliced LTR-driven RNAs, respectively.

cedure described previously (44). After several PBS rinses and an incubation in 3% hydrogen peroxide, the sections were placed in a 5% normal goat serum. The sections were then incubated in the primary anti-GFP antibody (1:1,000; Clontech, Palo Alto, Calif.) in 1% normal goat serum–0.1% Triton X-100 overnight at room temperature. After rinsing, the sections were incubated in the biotinylated rabbit anti-goat secondary antibody (Vector, Burlingame, Calif.) for 3 h. After rinsing, the sections were incubated with horseradish peroxidase-streptavidin and then reacted by using a purple chromagen kit (VIP; Vector), mounted, dried, dehydrated, and coverslipped.

## RESULTS

Tat is required to produce a vector of efficient transducing activity. To investigate the role of Tat in the production of transducing particles, expression from lentivirus vectors was first examined by Northern analysis (Fig. 1). The patterns of RNAs induced by transfer vectors in which the transgene was driven by an internal PGK, CMV, or retrovirus MFG promoter were studied in both producer and target cells. In transfected 293T cells, expression occurred mainly from the internal promoter. When a packaging construct expressing both Tat and Rev was cotransfected, a dramatic enhancement of transcription from the LTR was observed, with an accumulation of unspliced vector RNA. In cells transduced with the vectors, that is, in the absence of Tat and Rev, transcription from the LTR was almost completely suppressed, the residual transcripts underwent splicing, and the internal promoter was responsible for most of the expression.

A packaging plasmid carrying two mutations in *wt* (pCMVAR.93) was then constructed. The first mutation is a deletion of the T in the ATG initiation codon of the *tat* gene; the second is an insertion of a *Mut* linker producing a translation stop codon after residue 46 of the Tat protein. These changes confer a *wt*-defective phenotype to HIV-1 (12). After transfection of the control or *wt*-defective packaging constructs into 293T cells, comparable yields of vector particles were recovered in the culture medium, as assayed by using the Gag p24 antigen (see Table 3). Such Tat independence was expected from the replacement of the HIV LTR by the constitutive CMV promoter in the packaging construct. However, the particles produced in the absence of Tat had a dramatically reduced transducing activity (Table 1): 5 to 15% of that of particles produced by the control Tat-positive packaging construct.

We also tested whether the Tat-defective phenotype could be rescued by complementation in target cells (Table 1). HeLa-tat cells, a cell line expressing Tat from the HIV-1 LTR (13), were transduced by vectors produced with or without Tat. The expression of Tat in target cells did not compensate for the loss in transduction efficiency of vector produced without Tat.

As expected from the Northern analysis, functional inactivation of the *wt* gene resulted in a lower abundance of vector RNA in producer cells. This was indicated by the decrease in luciferase activity in cells producing a luciferase vector without an internal promoter. In this case, transgene expression di-

TABLE 1. Transducing activities of lentivirus vectors made with and without a functional *wt* gene in the packaging construct<sup>a</sup>

Transfer vector	Target cells	Mean transducing activity (TU/ng of p24) ± SE <sup>b</sup>	
		With <i>wt</i> in packaging construct	Without <i>wt</i> in packaging construct
pH2'CMV-LacZ	293T	1,056 ± 54	152 ± 26
pH2'PGK-eGFP	HeLa	5,066	384
pH2'CMV-Luciferase	HeLa	3,000 ± 152	152 ± 26
	HeLa-tat	3,777 ± 348	486 ± 59
pH2'Luciferase <sup>c</sup>	HeLa	46 ± 1	0.3 ± 0.003
	HeLa-tat	3,296 ± 276	174 ± 75

<sup>a</sup> Vectors were produced by transfection of the indicated transfer vector, a packaging construct either with (pCMVAR.91) or without (pCMVAR.93) a functional *wt* gene, and plasmid pMD.G into 293T cells. Serial dilutions of transfected conditioned medium were incubated with the indicated cells, and the cultures were scored after 3 days. For calculating transduction activity, samples were selected from the linear portion of the vector dose-response curve.

<sup>b</sup> LacZ transduction was measured by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining and by expression of the number of blue cell colonies as a function of the amount of p24 antigen in the inoculum. eGFP transduction was measured by FACS analysis, multiplying the fraction of fluorescent cells by the number of infected cells, and expressing the result as a function of the amount of p24 antigen in the inoculum. Luciferase transduction was measured by luminescence in RLU above background of 50 µl of culture extract and dividing the number of RLU × 10<sup>-2</sup> by the number of nanograms of p24 antigen in the inoculum. Means of duplicate (pH2'PGK-eGFP) or triplicate (all other constructs) determinations are shown.

<sup>c</sup> Without internal promoter.

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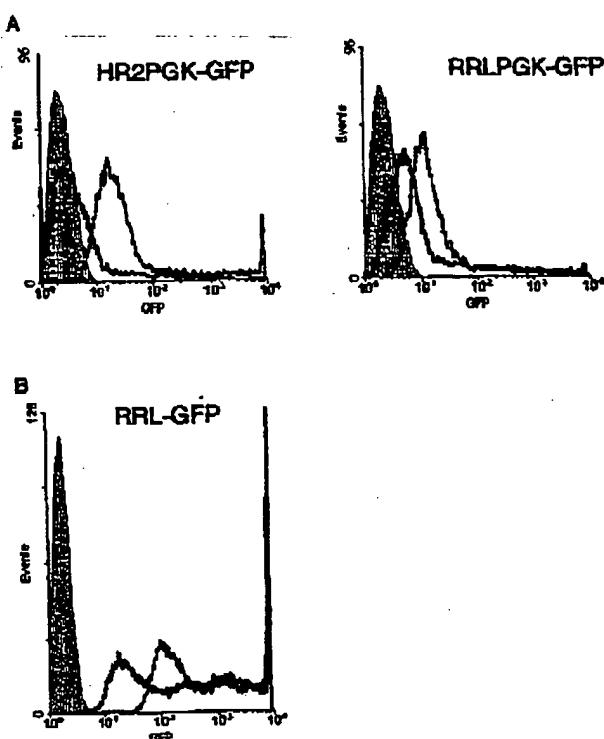


FIG. 2. Transcriptional activities of wild-type and 5' chimeric vector constructs in the absence and presence of Tat. (A) Control pHR2 and the 5' chimeric pRRL transfer construct carrying a PGK-eGFP expression cassette were transfected into 293T cells with a packaging construct having a functional (pCMVAR8.91; grey line) or inactive (pCMVAR8.93; black line) *tat* gene. GFP expression was analyzed by FACS. The filled area represents nontransfected cells. In the absence of Tat, the chimeric construct yielded a level of GFP expression higher than that achieved by the pHR2 construct. Both constructs were further upregulated by Tat. (B) A pRRL construct carrying the eGFP gene without an internal promoter was transfected with a packaging construct carrying a functional (grey line, open area) or inactive (black line, open area) *tat* gene. Direct upregulation of the chimeric promoter by Tat was observed. The filled area represents nontransfected cells.

rectly reflects the abundance of transcripts originating from the LTR. 293T cells producing luciferase vectors without Tat had only 5% of the luciferase content of cells producing the same vector with Tat ( $[1.0 \pm 0.2] \times 10^6$  relative light units [RLU]/dish without Tat;  $[20.2 \pm 0.7] \times 10^6$  RLU/dish with Tat). This ratio corresponded very closely to that observed in cells transduced by either type of vector in the course of the same experiment (Table 1), suggesting that the abundance of vector RNA in producer cells is a rate-limiting factor in the transduction by lentivirus vectors.

One could thus conclude that Tat is required in producer cells to activate transcription from the HIV LTR and to generate vector particles with a high transducing activity.

The *tat* requirement is offset by placing a constitutive promoter upstream of the transfer vector. If the only function of Tat is *trans* activation of vector transcription from the LTR, the *tat*-defective phenotype should be rescued by placing a strong constitutive promoter upstream of the vector transcript. Three transcriptional domains have been identified in the HIV promoter in the U3 region of the LTR: the core or basal domain, the enhancer, and the modulatory domain (27). Transcription starts at the U3/R boundary, the first nucleotide of R being numbered 1. The core promoter contains binding sites for the

TATA-binding protein (-28 to -24) and SP-1 (three binding sites between -78 to -45). The enhancer contains two binding sites for NF- $\kappa$ B which overlap with a binding site for NFATc (-104 to -81). The modulatory domain contains binding sites for several cellular factors, including AP-1 (-350 to -293), NFAT-1 (-256 to -218), USF-1 (-166 to -161), Ets-1 (-149 to -141), and LEF (-136 to -125). A panel of 5' chimeric transfer constructs carrying substitutions of either all or part of the U3 region of the 5' LTR was generated. All substitutions were made to preserve the transcription initiation site of HIV. Partial substitutions joined new enhancer sequences to the core promoter of the HIV LTR (-78 to 1), while full substitutions replaced also the promoter. pRLL and pRRL vectors carried the enhancer and the enhancer/promoter, respectively, of RSV; pCCL and pCCL vectors carried the enhancer and the enhancer/promoter of human CMV.

Control pHR2 and 5' chimeric transfer constructs carrying a PGK-eGFP expression cassette were tested by transfection of 293T cells with control or *tat*-defective packaging constructs, and the expression of the eGFP transgene was analyzed by fluorescence-activated cell sorting (FACS). The RRL chimeric construct yielded a higher level of eGFP expression than the pHR2 vector, reflecting the constitutive transcriptional activity of the new sequence (Fig. 2A). Interestingly, the chimeric vector also displayed upregulation by Tat, as shown by the increased eGFP expression of cells cotransfected with the control packaging construct. Tat upregulation was proven to be a direct effect by transfecting a pRRL-eGFP vector lacking an internal promoter with control or *tat*-defective packaging constructs and analyzing GFP expression by FACS (Fig. 2B). Comparable results were obtained with the other chimeric LTR vectors (not illustrated). Vector particles were then collected from the transfected producer cells and assayed for transduction of eGFP into HeLa cells and human primary lymphocytes (peripheral blood lymphocytes [PBL]). As shown in Table 2, all vectors had efficient transducing activity, as assessed by endpoint titration on HeLa cells or maximal transduction frequency of PBL. The vector produced by the pRRL chimera was as efficient as that produced by the pHR2 construct and was selected to test transduction independent of Tat. As shown in Table 3, the pRRL construct yielded a vector of only slightly reduced transducing activity (60%) when the packaging construct was *tat* defective. The residual effect of

TABLE 2. GFP transduction by lentivirus vectors made by transfer constructs with a wild-type or 5' chimeric LTR

Transfer construct	Endpoint titr on HeLa cells (TU/ml) <sup>a</sup>	Transduction efficiency on human lymphocytes (% positive cells) <sup>b</sup>
pHR2	$2.3 \times 10^7$	30
pCCL	$4.6 \times 10^6$	14
pCCL	$7.9 \times 10^6$	18
pRRL	$1.8 \times 10^7$	29
pRRL	$8.9 \times 10^6$	18

<sup>a</sup> Determined by multiplying the percentage of fluorescent cells for the vector dilution and the number of infected cells. Samples were selected from the linear portion of the vector dose-response curve.

<sup>b</sup> Percentage of fluorescent human PBL after infection of  $10^6$  cells with 1 ml of vector containing medium. Primary human T lymphocytes were isolated and transduced as previously described (14). Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer construct, the packaging plasmid pCMVAR8.91, and the envelope plasmid pMO.G into 293T cells. Fluorescent cells were scored by FACS analysis 6 days after transduction. Data are averages of duplicate determinations for a representative experiment of three performed.

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TABLE 3. GFP transduction into HeLa cells by lentivirus vectors made by transfer constructs with a wild-type or 5' chimeric LTR and packaging constructs with or without a functional *tar* gene\*

Transfer construct	<i>tar</i> gene in packaging construct	Endpoint titer (TU/ml)	p24 antigen (ng/ml)	Transduction efficiency (TU/mg of p24)
pHR2	+	$4.1 \times 10^6$	297	13,805
pHR2	-	$2.4 \times 10^5$	545	440
pRRL	+	$1.3 \times 10^7$	546	23,810
pRRL	-	$4.9 \times 10^6$	344	14,244

\* Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer and packaging plasmid plus plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determinations for a representative experiment of five performed.

Tat on transduction was in agreement with the ability of Tat to upregulate transcription from the chimeric LTR.

The use of the chimeric LTR construct allowed removal of Tat from the packaging system with a minimal loss in the

transduction efficiency of the vector *in vitro*. To test vector performance in the more challenging setting of *in vivo* delivery into brain neurons, high-titer vector stocks were generated from the pHR2 and pRRL constructs with and without Tat. The four stocks of eGFP vector were matched for particle content by p24 antigen and injected bilaterally in the neostriata of groups of three adult rats. The animals were sacrificed after 1 month, and serial sections of the brain were analyzed for eGFP fluorescence (not shown) and immunostained by antibodies against eGFP (Fig. 3). The results obtained *in vivo* matched the *in vitro* data. Vector produced by the pHR2 construct only achieved significant transduction of the neurons when packaged in the presence of Tat. Vector produced by the pRRL chimera was as efficient when made with or without Tat. The transduction extended throughout most of the striatum and reached a very high density of positive cells in the sections closest to the injection site. No signs of pathology were detectable in the injected tissue, except for a small linear scar marking the needle track, by hematoxylin and eosin staining of the sections (data not shown).

These results provide evidence that Tat is dispensable for efficient transduction by a lentivirus vector.

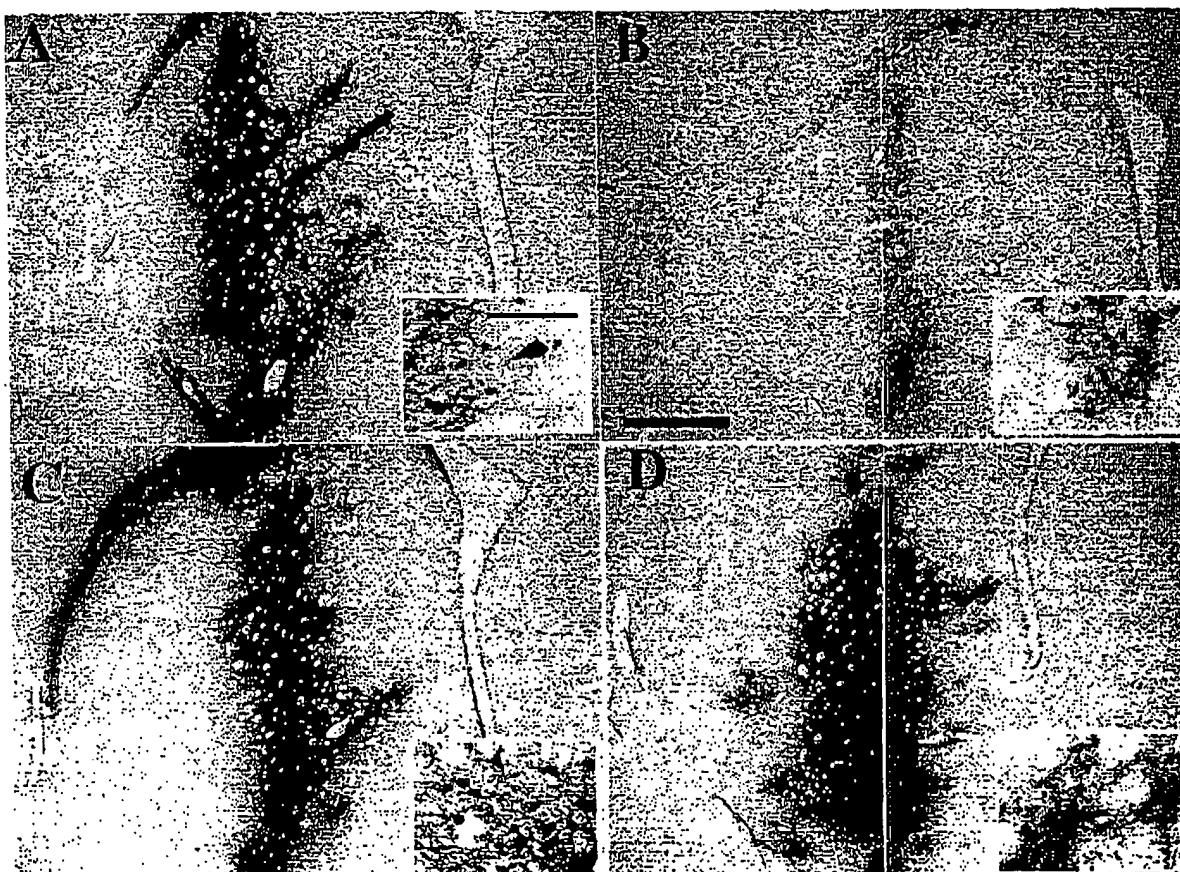


FIG. 3. *In vivo* transduction of eGFP into brain cells by lentivirus vectors produced with and without Tat. Vectors carrying a PGK-eGFP expression cassette were produced by the pHR2 (A and B) or the 5' chimeric pRRL (C and D) transfer construct and a packaging construct with (pCMVΔR8.91; A and C) or without (pCMVΔR8.93; B and D) a functional *tar* gene, concentrated by ultracentrifugation, and normalized for particle content prior to injection into the corpus striatum of adult rats. One month after injection, brain sections were stained for immunoreactivity to the GFP protein. While both types of vectors transduced neurons very efficiently when made with Tat, only the vector made by the chimeric transfer construct worked as well when produced without Tat. Representative sections close to the injection site are shown for one of six striata injected per each type of vector. The bar in panel B represents 1 mm; that in the inset in panel A represents 100  $\mu$ m.

TABLE 4. GFP transduction into HeLa cells by lentivirus vectors made by linked or split packaging constructs and a pRRL transfer construct<sup>a</sup>

Packaging construct	Separate <i>rev</i> plasmid <sup>b</sup>	p24 antigen (ng/ml)	Endpoint titer (TU/ml)	Transduction efficiency (TU/ng of p24)
pCMV $\Delta$ R8.74		364	1.07 × 10 <sup>7</sup>	29,436
pMDLg/pRRE		<0.1	ND	NA
pMDLg/pRRE	TK-Rev, 5 $\mu$ g	29	6.9 × 10 <sup>6</sup>	23,793
pMDLg/pRRE	TK-Rev, 12 $\mu$ g	94	2.02 × 10 <sup>6</sup>	21,499
pMDLg/pRRE	RSV-Rev, 2.5 $\mu$ g	774	1.0 × 10 <sup>7</sup>	13,495
pMDLg/pRRE	RSV-Rev, 5 $\mu$ g	776	7.6 × 10 <sup>6</sup>	9,761
pMDLg/pRRE	RSV-Rev, 12 $\mu$ g	565	4.8 × 10 <sup>6</sup>	8,495

<sup>a</sup> Vectors carrying a PGK-cGPP expression cassette were produced by the transfection of a self-inactivating pRRL transfer construct (with a deletion in the 5' LTR [35]), the indicated packaging and *rev* plasmids, and plasmid pMD.G into 293T cells. Serial dilutions of transfected conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determination for a representative experiment of three performed. ND, none detected (the detection limit of the assay was 10<sup>5</sup> TU/ml); NA, not applicable.

<sup>b</sup> The promoter driving the expression of a synthetic *rev* cDNA and the amount of plasmid transfected are indicated.

**A new split-genome conditional packaging system.** The possibility of deleting the *tat* gene prompted us to explore a new design of the packaging component of the HIV vector system, in which two separate nonoverlapping expression plasmids, one for the *gag* and *pol* genes and the other for the *rev* gene, were used. The *gag* and *pol* reading frames were expressed within the context of the MD cassette, which employs the CMV promoter and intervening sequence and the human  $\beta$ -globin poly(A) site (34). All HIV sequences upstream of the *gag* initiation codon were removed, and the leader was modified for optimal fit to the Kozak consensus for translation. This construct, however, expressed almost no p24 antigen when transfected alone in 293T cells. This observation is in agreement with the previously reported presence of *cis*-repressive or inhibitory sequences in the *gag* and *pol* genes (40, 41). The HIV RRE was then inserted downstream of the *pol* gene, and the resulting plasmid was cotransfected with a *rev* expression vector (Table 4). High levels of p24 antigen production were observed in this case, the highest yields being obtained when *rev* was driven by an RSV promoter. When the *gag-pol* and the *rev* constructs were cotransfected with the pRRL chimeric transfer vector and the VSV G-expressing plasmid, high-titer vector was obtained in the culture medium. Both the yield of particles and their transducing efficiency were similar to those obtained with previous versions of the system. Northern analysis of producer cells confirmed that unspliced vector genomic RNA accumulated only in the presence of Rev (data not shown). Thus, both the expression of the *gag* and *pol* genes and the accumulation of packageable vector transcripts are dependent on *trans* complementation by a separate Rev expression construct. Such a conditional packaging system provides an important safety feature unavailable to oncoretrovirus vectors.

## DISCUSSION

The predicted biosafety of a viral vector depends in part on how much segregation of the *cis*- and *trans*-acting functions of the viral genome is achieved by the vector design and is maintained during vector production. A vector particle is assembled by viral proteins expressed in the producer cell from a construct(s) stripped of the *cis*-acting sequences required for the transfer of the viral genome to target cells (packaging con-

struct). These *cis*-acting sequences are instead linked to the transgene in the transfer vector. As the vector particle packages only the genetic information contained in this latter construct, the infection process is limited to a single round without spreading. Through recombination, it is possible that sequences encoding viral proteins rejoin the *cis*-acting elements of the transfer vector. If the resulting recombinant expresses all required functions, it is able to replicate (i.e., it is a replication-competent retrovirus [RCR]) and presents a risk to the recipient. The formation of heterozygous vector particles containing RNAs from both the packaging and transfer vectors, followed by homologous recombination during reverse transcription, is the mechanism most often incriminated in the emergence of RCR during the production of retroviral vectors. The likelihood of this type of recombination is dependent on residual *cis*-acting sequences in the packaging plasmid, allowing some level of encapsidation, and on the extent of homology between packaging and vector constructs (10).

A first strategy to improve the biosafety of a vector is to use nonoverlapping split-genome packaging constructs that require multiple recombination events with the transfer vector for RCR generation. Earlier studies described several approaches to generate replication-defective HIV vectors (7, 35, 38, 42). However, these vectors could be produced only to low infectious titers, were restricted to CD4-positive cellular targets, and carried the risk of generating wild-type HIV by recombination of the components. A major advance was achieved when an improved vector design was combined with the use of the envelope of another virus (32, 33, 39). The lentivirus vector that we describe here is packaged by three nonoverlapping expression constructs, two expressing HIV proteins and the other expressing the envelope of a different virus. Moreover, all HIV sequences known to be required for encapsidation and reverse transcription (2, 22, 24, 27, 29, 30, 35) are absent from these constructs, with the exception of the portion of the *gag* gene that contributes to the stem-loop structure of the HIV-1 packaging motif (29).

A second strategy to improve vector biosafety took advantage of the complexity of the lentivirus genome. The minimal set of HIV-1 genes required to generate an efficient vector was identified, and all other HIV reading frames were eliminated from the system. As the products of the removed genes are important for the completion of the virus life cycle and for pathogenesis, no recombinant can acquire the pathogenetic features of the parental virus. We previously demonstrated that all four accessory genes of HIV could be deleted from the packaging construct without compromising gene transduction (51). In this work, we went further by deleting another factor crucial for HIV replication, the *tat* gene. Its product is one of the most powerful transcriptional activators known and plays a pivotal role in the exceedingly high replication rates that characterize HIV-induced disease (18, 19, 47).

It was found that Tat was required in producer cells to generate vector of efficient transducing activity but that this requirement was offset by inducing constitutive high-level expression of vector RNA. Due to the low basal transcription from the HIV LTR, Tat was necessary to increase the abundance of vector transcripts and allow their efficient encapsidation by the vector particles. When made in the absence of Tat, vector particles had 10- to 20-fold-reduced transducing activity. However, when strong constitutive promoters replaced the HIV sequence in the 5' LTR of the transfer construct, vectors made without Tat exhibited a less than twofold reduction in transducing activity. As Tat strongly upregulated transcription from the chimeric LTR, the transducing activity of the output particles must reach saturation. The abundance of vector RNA

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in producer cells thus appears to be a rate-limiting factor for transduction until it reaches a threshold. Conceivably, an upper limit is set by the total output of particles available to encapsidate vector RNA. As the total particle output varied with the types of vector and internal promoter used, this may explain the quantitative differences obtained in response to *tat* deletion.

Successful deletion of the *tat* gene was unexpected in view of a reported additional role for Tat in reverse transcription (17, 20). While the reasons for this discrepancy are not obvious, it should be noted that the transduction pathway of the lentivirus vector mimics only in part the infection pathway of HIV. The vector is pseudotyped by the envelope of an unrelated virus and contains only the core proteins of HIV, without any accessory gene product. The VSV envelope targets the vector to the endocytic pathway, and it has been shown that redirection of HIV-1 from its normal route of entry by fusion at the plasma membrane significantly changes the biology of the infection. For example, Nef and cyclophilin A are required for the optimal infectivity of wild-type HIV-1 but not of a (VSV G) HIV pseudotype (1). It is also possible that the kinetics of reverse transcription are more critical for the establishment of viral infection than for gene transduction, given the differences in size and sequence between the virus and vector genome.

Tat-independent transduction by an HIV-based vector was recently reported by Kim et al. for *in vitro* cellular targets (23). In the vector designed by these authors, however, Tat and Rev were expressed from the transfer vector and thus were also present in target cells. A CMV-HIV hybrid LTR was used; this construct yielded vector titers approximately 30% of that obtained with an intact LTR. When the *tat* gene was inactivated, the titer did not change. Srinivasakumar et al. (43) previously reported a rather low (5- to 10-fold) dependence on Tat of an HIV-based vector produced by cells stably expressing the HIV structural proteins. In this case, titers of  $5 \times 10^3$  TU/ml with Tat and  $7 \times 10^2$  TU/ml without Tat were obtained on HeLa-CD4 cells. Although these titers are much lower than those reported here, the vector particles carried the HIV envelope, an indication that Tat is not absolutely required for transduction by vector particles which in that case mirror more closely the wild-type virus. It remained possible, however, that a dependence on Tat may be revealed in more challenging gene deliveries into the body tissues that are the actual targets of gene therapy. This could have been due to a stricter Tat requirement for optimal transduction efficiency or for the production of high-titer vector stocks or to differences in cell-type-specific factors. Our results now establish that Tat is fully dispensable for lentivirus vector transduction even when high titers are achieved and, most importantly, for gene delivery *in vivo* into terminally differentiated neurons of an adult rat brain.

The Northern analysis of producer and target cells shows that the Tat dependence of LTR-driven expression restricts the production of vector genomic RNA to producer cells. This applies as well to vectors made by the 5' chimeric constructs, as the U3 sequences of both LTRs of the resulting provirus are derived from the vector 3' LTR. However, the functional replacement of the *tat* gene in the packaging construct by promoter sequences upstream of the transfer construct makes the generation of a transcriptionally active recombinant much more unlikely. This will be even more significant in stable producer cell lines that avoid the risk of plasmid recombination during cotransfection.

We also exploited the Rev dependence of *gag-pol* expression and of the accumulation of unspliced, packageable transcripts. Yu et al. (50) previously showed that the dependence on Rev can be used to make expression of HIV genes inducible. We

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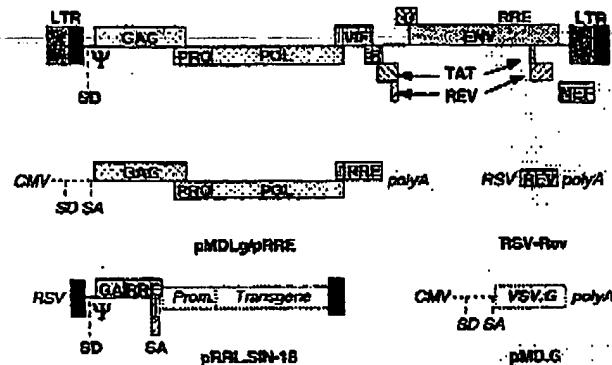


FIG. 4. Schematic drawing of the HIV provirus and the four constructs used to make a lentivirus vector of the third generation. The viral LTRs, the reading frames of the viral genes, the major 5' splice donor site (SD), the packaging sequence (p), and the RRE are boxed and indicated in bold type. The conditional packaging construct, pMDLg/pRRE, expresses the gag and pol genes from the CMV promoter and intervening sequences and polyadenylation site of the human β-globin gene. As the transcripts of the gag and pol genes contain cis-repressive sequences, they are expressed only if Rev promotes their nuclear export by binding to the RRE. All tat and rev genes have been deleted, and the viral sequences upstream of the gag gene have been replaced. A nonoverlapping construct, RSV-Rev, expresses the rev cDNA. The transfer construct, pRRL-SIN-18, contains HIV-1 cis-acting sequences and an expression cassette for the transgene. It is the only portion transferred to the target cells and does not contain wild-type copies of the HIV LTR. The 5' LTR is chimeric, with the enhancer/promoter of RSV replacing the U3 region (RRR) to rescue the transcriptional dependence on Tat. The 3' LTR has an almost complete deletion of the U3 region, which includes the TATA box (from nucleotides -418 to -18 relative to the U3/R border). As the latter is the template used to generate both copies of the LTR in the integrated provirus, transduction of this vector results in transcriptional inactivation of both LTRs; thus, it is a self-inactivating vector (SIN-18). The fourth construct, pMDL.G, encodes a heterologous envelope to pseudotype the vector, here shown coding for VSV G. Only the relevant parts of the constructs are shown.

describe a core packaging system split in two separate nonoverlapping expression constructs, one for the gag and pol reading frames optimized for Rev-dependent expression and the other for the rev cDNA. This third-generation packaging system matches the performance of its predecessors in terms of both yield and transducing efficiency. However, it increases significantly the predicted biosafety of the vector. It has been suggested that the Rev-RRE axis could be replaced by the use of constitutive RNA transport elements of other viruses, although at the price of decreased efficiency (11, 23, 43). We would suggest that maintaining the Rev dependence of the system allows for an additional level of biosafety through the splitting of the HIV-derived components of the packaging system.

The conditional packaging system described here can be combined with a self-inactivating vector construct carrying a major deletion in the 3' LTR (52). This vector design (Fig. 4) offers significant biosafety features. The contribution of HIV is reduced to a fraction of cis-acting sequences in the vector, leaving out in particular most of the LTR, and to only three genes, gag, pol, and rev, in the packaging constructs, compared with the nine genes necessary for the *in vivo* replication and pathogenesis of wild-type HIV-1 (3, 18, 27, 49). The actual biosafety of a vector must be proven *in vivo*. However, given the serious limitations of the available animal models of HIV-induced disease, the biosafety of HIV-derived vectors will ultimately be proven only in human hosts. Therefore, the vector design must ensure the highest predictable biosafety for clinical testing to be acceptable.

It is noteworthy that the fraction of the HIV-1 genome that is left in the vector is probably smaller than could be achieved

with any of the nonprimate lentiviruses, the genomic complexity of which is lower than that of HIV-1 (37). Also, the risks associated with the introduction in humans of a recombinant arising from a nonprimate lentivirus, even in a form that in its cognate animal species appears to be attenuated, are very difficult to assess, as illustrated by the ongoing debate on xenotransplantation (48). In contrast, the almost two decades spent studying a virus that has now spread in tens of millions of people worldwide have revealed a considerable amount of information on the pathogenic features of HIV-1, in particular on the dependence of virulence on a crucial set of viral genes. Based on these data, we would like to suggest that the HIV-based vectors described here are good candidates for the clinical trial of lentivirus vectors in human gene therapy.

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